Prevalence of Asymptomatic and/or Low-Density Malaria Infection among High-Risk Groups in Peninsular Malaysia

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Abstract. Asymptomatic and/or low-density malaria infection has been acknowledged as an obstacle to achieving a malaria-free country. This study aimed to determine the prevalence of asymptomatic and/or low-density malaria infection in previously reported malarious localities using nested PCR in four states, namely, Johor, Pahang, Kelantan, and Selangor, between June 2019 and January 2020. Blood samples ($n = 585$) were collected and were extracted using a QIAamp blood kit. The DNA was concentrated and subjected to nested PCR. Thin and thick blood smears were examined as well. Of the 585 samples collected, 19 were positive: 10 for *Plasmodium knowlesi*, eight for *Plasmodium vivax*, and one for *Plasmodium ovale*. Asymptomatic and/or low-density malaria infection is a threat to malaria elimination initiatives. Eliminating countries should develop guidance policy on the importance of low-density malaria infection which includes detection and treatment policy.

Besides China and the Republic of Korea, Malaysia is on course to achieve malaria-free status by the end of 2020.¹ However, the efforts committed to achieve this status are dampened because of high proportion of *Plasmodium knowlesi* malaria cases reported in Malaysian Borneo² and also in Peninsular Malaysia.³ *Plasmodium knowlesi* malaria has been well acknowledged and recognized as the predominant contributor to malaria cases in Malaysia,^{2,3} which may cause severe disease and has a fatality rate similar to that of *Plasmodium falciparum* infections.²

Asymptomatic and/or low-density malaria infection has been known for many years and is a result of premunition, partial immunity toward the parasite that controls the infection from becoming severe but does not obliterate the parasite.⁴ The term asymptomatic applies to individuals who are harboring the parasite but presenting no specific clinical manifestations of the infection. Low-density infection on the other hand implies toward infection in which the parasitemia is not being quantified via conventional diagnostic tests such as blood film malaria parasite (BFMP) or rapid diagnostic tests. Rather, this low-density infection is identified with the utilization of a highly sensitive diagnostic method, namely, PCR.⁵

Asymptomatic and/or low-density malaria infection is thus far an imperative obstacle in reducing the number of malaria cases worldwide as it also contributes to the transmission of *P. knowlesi*.⁶ It was evident from previous studies that mosquitoes can be infected by biting children with submicroscopic gametocytemia. Although infectivity is lower than microscopic gametocytemia infections, transmission of submicroscopic infection to a mosquito serves as an important precursor for subsequent infections.⁷ The gold standard of malaria diagnosis is BFMP. However, because of its low detection limit, a vast array of molecular methods has been implemented to supplement the BFMP results. Of these, nested PCR or real-time PCR is widely used with a detection limit of 100–1,000 parasites per milliliter of blood⁸ as

compared with 10,000–100,000 parasites per milliliter of blood for BFMP.⁹

This study aimed to screen for the presence of low-density malaria infection among asymptomatic communities living in high-transmission areas of malaria in Peninsular Malaysia via a molecular method. A total of 667 and 760 malaria cases were reported in Peninsular Malaysia in 2017 and 2018, respectively (Ministry of Health Malaysia, unpublished data). This cross-sectional study has covered four states with the highest incidence rate of malaria in Peninsular Malaysia, Johor (81 and 64 cases in 2017 and 2018, respectively), Pahang (85 and 123 cases in 2017 and 2018, respectively), Kelantan (168 and 135 cases in 2017 and 2018, respectively), and Selangor (105 and 102 cases in 2017 and 2018, respectively), and was conducted from June 2019 to January 2020. Sampling was conducted in districts with previously reported malaria cases including Mersing, Kota Tinggi, and Kluang in Johor; Kuala Lipis, Maran, and Jerantut in Pahang; Gua Musang in Kelantan; and Hulu Selangor and Hulu Langat in Selangor. These districts have been selected as the cases of malaria reported were high in 2017 and 2018 (Ministry of Health Malaysia, unpublished data), and thus, this study hypothesized the presence of asymptomatic and/or low-density malaria infection among the communities of high-risk groups. Here, a high-risk group is defined as individuals working in proximity with forest and forest fringes, for instance, servicemen, farmers, hunters, and natural resource collectors as described previously.^{10,11} Close-contact individuals in the same village of previously reported cases were also recruited voluntarily in this study. In brief, the study sites were selected based on previously reported malaria incidence, whereas the respondents were recruited based on close proximity with the index's house (typically of the same village) as well as high-risk group individuals.

Approximately, 5 mL of whole blood from consenting respondents was collected in a heparinized blood collection tube for analysis via nested PCR and blood film examination. Demographic data of the respondents, history of experiencing any symptoms of malaria, previous malarial infection, and visit to forest or agricultural areas were collected as well. This study has been approved by the Medical Research Subcommittee of the Malaysian Ministry of Health (NMRR-15-67223975).

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Thin and thick blood films were prepared using a few drops of heparinized whole blood within 5 hours of sample collection. Thin blood films were fixed with methanol and stained immediately with 10% Giemsa for 20 minutes and air-dried. Thick films were air-dried overnight before staining with 10% Giemsa. For extraction of DNA, 1 mL of whole blood was centrifuged at $1,200 \times g$ for 10 minutes to remove plasma and buffy coat. The packed RBC (pRBC) was then stored in liquid nitrogen temporarily (1–5 days) and then frozen at -80°C . Two hundred to 600 μL of pRBC was extracted using a QIAamp blood mini kit or QIAamp blood midi kit (Qiagen, Hilden, Germany). The extracted DNA was concentrated until 10 μL of sample left in the microcentrifuge tube. The equivalent of 200 μL of whole blood was screened using nested PCR.

Extracted DNA was subjected to nested PCR assays targeting the *Plasmodium* 18S small subunit ribosomal RNA in which the primer pair rPLU1 and rPLU5 was used in nest 1, whereas primer pair rPLU3 and rPLU4 was used in nest 2.⁸ Should positive amplification observed on the second round of amplification, the product of the first round amplification would be subjected to species detection using species-specific primer pairs: rFAL1 and rFAL2, rMAL1 and rMAL2, rVIV1 and rVIV2, rOVA1 and rOVA2,⁸ and PkF1140 and PkR1550.¹² The cycle profiling used was as per described.¹² Products of nest 2 were electrophoresed and visualized. Species-specific PCR products were directly ligated into pGEM[®]-T (Promega, WI) and transformed into One Shot[™] TOP10F' Chemically Competent *Escherichia coli* (Invitrogen). Positive recombinant plasmids were sent to First BASE Laboratories Sdn. Bhd., Malaysia, and DNA sequences were then subjected to BLAST analysis for confirmation of species identity. Then, re-extraction of positive samples was performed for loop-mediated isothermal amplification (LAMP) analysis to further validate the results.

A total of 585 blood samples were collected from June 2019 to January 2020, of which 217 blood samples were collected from army personnel. The demographic data of the study locations are shown in Table 1. Majority (73.8%) of the respondents were males ($n = 432$), and 79.0% ($n = 462$) of the respondents were in the 15–45 years age-group. Most of the respondents were healthy (96.1%, $n = 562$), not experiencing any signs and symptoms for malaria, specifically high fever, chills, tremors, or headaches. The rest of the respondents (3.9%) self-reported fever and/or headache, but on nested PCR, they were negative for malaria. A small proportion (5.8%, $n = 34$) had acquired malaria previously, and 67.2% ($n = 397$) stated that they had been to malaria-prone areas such as jungle fringes, rubber, and palm oil plantations. Of the 585 samples, 19 (3.3%) were positive for malaria parasites as tested by nested PCR (Table 2). All subjects who were positive, 19 (100%), sustain asymptomatic and low-density infection. *Plasmodium knowlesi* was found to be the predominant species (52.6%; $n = 10$) followed by *Plasmodium vivax* (42.1%; $n = 8$). *Plasmodium ovale* (5.3%; $n = 1$), although rare in Malaysia, was also found in this study. None of the samples were positive when tested by BFMP. Eleven (57.9%) of the positive respondents were army personnel who frequented forests during training, and 16 (84.2%) of the 19 positive respondents did visit forest or agricultural areas before 3 months from the date of blood collection.

A cohort study on untreated asymptomatic *P. falciparum* infection among west Kenyan children by Bousema et al. reported patients with gametocytemia in the absence of treatment and thus, acting as a disease reservoir.¹³ This finding is contradictory to the fundamental theory of the production of gametocytes. Differentiation from trophozoites to gametocytes in *P. falciparum* is generally governed by stimulants or stressors such as antimalarial drugs which target the asexual

TABLE 1
Demographic data of the study sites

Characteristic	Study population, % (No.)									Total % (No.)
	State									
	Johor			Pahang			Kelantan	Selangor		
	Kota Tinggi	Kluang	Mersing	Kuala Lipis	Maran	Jerantut	Gua Musang	Hulu Langat	Hulu Selangor	
No. of samples	26.5 (155)	17.3 (101)	2.8 (16)	11.1 (65)	2.1 (12)	3.4 (20)	24.4 (143)	8.0 (47)	4.4 (26)	100 (585)
Gender										
Male	72.9 (113)	99.0 (100)	100 (16)	55.4 (36)	75.0 (9)	50.0 (10)	64.3 (92)	95.7 (45)	42.3 (11)	73.8 (432)
Female	27.1 (42)	1.0 (1)	0	44.6 (29)	25.0 (3)	50.0 (10)	35.7 (51)	4.3 (2)	57.7 (15)	26.2 (153)
Age (years)										
15–45	74.8 (116)	99.0 (100)	100 (16)	75.4 (49)	41.7 (5)	45.0 (9)	84.6 (121)	66.0 (31)	57.7 (15)	79.0 (462)
46–60	9.7 (15)	0	0	10.8 (7)	16.6 (2)	25.0 (5)	9.1 (13)	6.4 (3)	27.0 (7)	8.9 (52)
> 60	3.2 (5)	0	0	13.8 (9)	41.7 (5)	30.0 (6)	6.3 (9)	0	15.3 (4)	6.5 (38)
Undetermined	12.3 (19)*	1.0 (1)*	0	0	0	0	0	27.6 (13)*	0	5.6 (33)
Self-reporting malaria symptoms										
Yes	2.6 (4)	99.0 (1)	6.3 (1)	4.6 (3)	8.3 (1)	15.0 (3)	5.6 (8)	0	7.7 (2)	3.9 (23)
No	97.4 (151)	1.0 (100)	93.7 (15)	95.4 (62)	91.7 (11)	85.0 (17)	94.4 (135)	100 (47)	92.3 (24)	96.1 (562)
Previous history of malaria infection										
Yes	1.9 (3)	0	6.3 (1)	12.3 (8)	8.3 (1)	20.0 (4)	9.8 (14)	0	11.5 (3)	5.8 (34)
No	98.1 (152)	100 (101)	93.7 (15)	87.7 (57)	91.7 (11)	80.0 (16)	90.2 (129)	100 (47)	88.5 (23)	94.2 (551)
Visit to forest or agricultural areas										
Yes	79.4 (123)	99.0 (1)	100 (16)	58.5 (38)	66.7 (8)	85.0 (17)	84.6 (121)	100 (47)	100 (26)	67.9 (397)
No	20.6 (32)	1.0 (100)	0	41.5 (27)	33.3 (4)	15.0 (3)	15.4 (22)	0	0	32.1 (188)

* Respondents did not provide the date of birth nor age.

TABLE 2
Species profile corresponding to the districts

Species	State								
	Johor			Pahang			Kelantan	Selangor	
	Kota Tinggi	Kluang	Mersing	Kuala Lipis	Maran	Jerantut	Gua Musang	Hulu Langat	Hulu Selangor
<i>Plasmodium falciparum</i>	0	0	0	0	0	0	0	0	0
<i>Plasmodium knowlesi</i>	6	3	1*	0	0	0	0	0	0
<i>Plasmodium vivax</i>	2	2	1	0	1	0	0	0	2
<i>Plasmodium malariae</i>	0	0	0	0	0	0	0	0	0
<i>Plasmodium ovale</i>	1	0	0	0	0	0	0	0	0

* The patient became febrile a few days post-sampling and was referred to a hospital.

erythrocytic stage of the parasite or other antipyretic drugs and herbal compounds.¹⁴ Similar study has not been carried out on *P. knowlesi* because of the failure in inducing gametocytogenesis toward in vitro-adapted *P. knowlesi* lines.¹⁵ Knowledge with regard to the development and longevity of *P. knowlesi* gametocytes in the human host is scarce. Gametocytes in the blood can be transmitted to other individuals, hence sustaining the transmission. In our study, the respondents were asymptomatic and most likely with low-density parasitemia; however, no parasite was seen under microscopic examination. Drug-induced release of gametocytes is unlikely as these individuals were not treated with antimalarials.

Our findings unveiled the presence of asymptomatic and/or low-density malaria infection among high-risk groups, such as military personnel, community in close proximity to jungle fringe, and people with a history of previous malaria infection. This study also found that asymptomatic and/or low-density malaria infection could occur not only in *P. vivax* malaria but also in *P. knowlesi* and *P. ovale* malaria. In Malaysia, only four studies^{7,16–18} on asymptomatic and/or low-density malaria infection were reported. *Plasmodium vivax*, followed by *P. knowlesi*, was the main parasite in asymptomatic and/or low-density malaria infection in Malaysian Borneo. Results in our study are consistent with previous studies^{6,17} where *P. knowlesi* accounted for majority of the positive cases. On the contrary, Jiram and her group found *P. vivax* as the predominant species in asymptomatic and/or low-density malaria infection in Belaga district, Kapit division, Sarawak.^{16,18} This could be due to the fact that these samples were collected during a *P. vivax* outbreak.¹⁸

Several countries in Southeast Asia have embarked on asymptomatic and/or low-density malaria infection-associated studies such as Indonesia,¹⁹ Myanmar, and Thailand.²⁰ These countries are categorized as low-transmission areas where malaria is hypoendemic.¹⁸ *Plasmodium vivax* was found to be the most prevalent species in these countries, and it poses unique challenges because the dormant hypnozoites can reactivate and differentiate into blood-stage merozoites and gametocytes. Asymptomatic *P. vivax* malaria is a common form of asymptomatic and/or low-density malaria infection in these low-transmission malarious areas. A limitation of this study is the small sample size; thus, our finding may not represent the whole population of high-risk groups in Peninsular Malaysia for asymptomatic and/or low-density malaria infection.

Partial immunity has been thought to be the possible reason for asymptomatic malaria infection. Such immunity is seen in individuals who are exposed to several genetically prominent clones of the parasite.²¹ In highly endemic malarious areas,

the locals are exposed to multiple clone inoculations, resulting in acute development of immunity against the disease as well as asymptomatic infections.²² Hence, throughout the infection, one is either asymptomatic as the individual clears up parasites in the blood, aided by antimalarial drugs, or is developing symptomatic infection.

Asymptomatic and/or low-density malaria infection could be an obstacle to the malaria elimination initiative by the Ministry of Health for several reasons. First, asymptomatic and/or low-density malaria infection could potentially be a source of sustained transmission in the community. Besides, detection of low-density malaria infection requires the use of highly sensitive methods such as molecular-based assay, and there has not been clear guidance policy on the use of molecular-based assay in detection of low-density malaria infection. It is important to note that there is no clear guidance policy on treatment of low-density malaria infection as well.

In brief, asymptomatic and/or low-density malaria infection is a threat to malaria elimination initiatives. Eliminating countries should develop guidance policy on the importance of low-density malaria infection which includes detection and treatment policy to fortify the modus operandi of eliminating malaria in this country.

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